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The Determination of Bilirubin with a New Enzymatic Method (Dri-STAT® Bilirubin) Using the Hitachi 704 Selective Analyzer

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Summary: A new enzymatic method for the determination of bilirubin in serum and plasma by means of a Hitachi 704 selective analyzer was evaluated. This endpoint method (37 °C) including a sample blank showed very reliable results. The range of linearity was 0.3 to 437 μmol/l bilirubin. The within-run imprecision of three different bilirubin concentrations (n = 16) was 0.37, 0.44 and 0.76% (coefficient of variation). Between-assay imprecision (n = 15) was 0.51 to 1.76% (coefficient of variation) for five different control materials. Inaccuracy, determined with 5 control sera (assigned values: 19, 23.9, 90.7, 142.0 and 295.6 μmol/l bilirubin), was 0.14 to 4.27%. Recovery rates, determined in two spiked plasma samples, were 97.8% and 99.1%, and in six bilirubin standard solutions between 92 and 99%. The comparison with the routinely used 2.5-dichlorophenyl diazonium salt method as well as with the *Jendrassik & Grof* ((1938) Biochem. Z. *297*, 81–89) method as the reference yielded correlation coefficients of r = 0.997 and r = 0.998.

Introduction

For several years, the determination of bilirubin has been one of the most frequently requested methods in clinical-chemical laboratories. Since *van den Bergh & Snapper* (1) first described the reaction of bilirubin in human serum with *Ehrlich*'s diazo reagent, a multitude of bilirubin methods has been published. Currently, many clinical laboratories use methods based on those of *Jendrassik & Grof* (2), or *Rand & di Pasqua*, using 2.4-dichlorophenyl diazonium salt (3), or *Wahlefeld* et al., using 2.5-dichlorophenyl diazonium salt (4) as the coupling reagent. In addition, bilirubin is sometimes determined by direct spectrometry (5).

The first enzymatic determination of bilirubin in serum was reported by *Grzesiak & Coleman* (6) and *Glick* et al. (7). They used bilirubin oxidase (EC 1.3.3.5), isolated and purified from *Myrothecium verrucaria* MT-1 by *Murao & Tanaka* (8) some years before. These authors had already discovered that oxidation of bilirubin (both conjugated and unconjugated) to biliverdin was catalysed by this enzyme, followed by a further oxidation of biliverdin to one or more purple compounds. The reaction sequence in the assay was:

$$\text{Bilirubin} + 1/2\ O_2 \xrightarrow[\text{oxidase}]{\text{Bilirubin}} \text{Biliverdin} + H_2O$$

$$\text{Biliverdin} + O_2 \longrightarrow \text{Purple compound(s)}$$

Recently this method has been jointly evaluated by three laboratories, using both the manual procedure and an adapted version on the centrifugal analyzer Cobas-Bio (9). The *Jendrassik & Grof* method was used as a reference.

The aim of the present study was to evaluate the method adapted to a Hitachi 704, using the *Jendrassik & Grof* procedure and the routinely used 2.5-dichlorophenyl diazonium salt method for comparison. Possible interfering substances were investigated.

Materials and Methods

Materials

For the precision and accuracy studies we used patient plasma samples (NH_4-heparinate) and control sera (Ultimate D, Decision 1, Beckman Instruments, München). For calibration of the 2.5-dichlorophenyl diazonium salt and the Dri-STAT method the C 8 calibrator (120.2 µmol/l bilirubin) from Beckman Instruments, München, was used. To prepare the plasma samples for evaluating the recovery rate we used bovine albumin-based bilirubin standards (No 7890, Sigma Chemie, München) dissolved in water to give final concentrations of 85.05 µmol/l (solution 1) and 350.6 µmol/l (solution 2), respectively.

Sample No 1:
1 part plasma (17.7 µmol/l bilirubin) + 4 parts bilirubin solution 1.

Sample No 2:
1 part plasma (176.0 µmol/l bilirubin) + 9 parts bilirubin solution 2.

Bilirubin (reference grade) was from Pfanstiehl Laboratories, Inc., Waukegan, IL 60085. Chemicals of p.a. quality for the *Jendrassik & Grof* reagents were from Sigma Chemie, München. Bovine serum albumin No. 11930 was from Serva, Heidelberg. Frigen® was from Raab & Karcher, München. The metabolic substances (reagent grade), ammonium chloride, urea, creatine, guanidine, guanidinosuccinic acid, uric acid, guanidinoacetic acid, xanthine, hypoxanthine, adenine, tryptamine, 3-indoxylsulphate (indican) and creatinine were from Sigma Chemie, München.

To study the influence of hypertriglyceridaemia 10 lipaemic sera (triacylglycerol concentrations 2.85 – 12.3 mmol/l) with low bilirubin (2.7 – 5.7 µmol/l) were split. For moderately elevated bilirubin values, one half of the samples was spiked with bilirubin (average concentration 30 µmol/l). The samples were then treated with Frigen® to separate the triacylglycerols according to *Voigt* (10). Bilirubin was measured in the samples before and after the clearing procedure.

Haemolysate was obtained by sonification of four times washed human erythrocytes followed by centrifugation.

To test metabolites for interference in the assay, the substances were added to aliquots of pooled human serum to obtain concentrations a hundred times higher than reference values found in healthy subjects (11).

Drugs and diagnostics were added to plasma in concentrations five times of those usually present in patients' sera.

Instruments

The 2.5-dichlorophenyl diazonium salt and the Dri-STAT® method were performed on a multichannel selective analyzer (Hitachi 704, Boehringer Mannheim, FRG). The instrument settings for the Dri-STAT® method are given in table 1. The *Jendrassik & Grof* method was performed manually on the Eppendorf Meßplatz MKE 5081 (Eppendorf Gerätebau, Hamburg, FRG).

Reagents

Reagents for the 2.5-dichlorophenyl diazonium salt method (Bilirubin DPD, No 123951) were from Boehringer Mannheim. The Dri-STAT®-bilirubin kit was from Beckman Instruments, München. Reagents and bilirubin standard solutions for the *Jendrassik & Grof* method were prepared as described by *Doumas* et al. (12).

Tab. 1. Instrument settings for the determination of total bilirubin (Hitachi 704)

Test	[EBili]
Assay Code	[2Point]:[15]–[32]
Sample Volume	[15]
R1 Volume	[350] [50] [NO]
R2 Volume	[30] [20] [NO]
Wavelength	[415] [546]
Calib. Method	[Linear] [0]
Std. (1) Conc.-Pos.	[0.0]–[1]
Std. (2) Conc.-Pos.	[120.1]–[3]
Unit	[µmol/l]
Sd Limit	[0.1]
Duplicate Limit	[100]
Sensitivity Limit	[0]
Abs. Limit (Inc/Dec)	[2000] [Decrease]
Expected Value	[0]–[600]
Instrument Factor	[1.00]

Methods

The Dri-STAT® method was performed according to the manufacturer's instructions, and the *Jendrassik & Grof* procedure, used as the reference method, was performed according to *Doumas* et al. (12, 13). The 2.5-dichlorophenyl diazonium salt method was also used for comparison, and was performed according to the manufacturer's instructions, except that the C8 calibrator (see 'materials') was employed.

Statistics

For statistical analysis of the paired samples in the method's comparison and of the method's linear range, a nonparametric procedure was employed (14). The calculations were performed on a desk top computer HP 217. For testing the influence of hypertriglyceridaemia on matched pairs, the *Wilcoxon* signed rank test (15) was used.

Results

Linearity

To study the linearity range, aliquots of a pool plasma were spiked with albumin-based bilirubin solutions of increasing bilirubin concentrations. Each dilution was determined in duplicate. We obtained linearity within the range of 0.3 – 437 µmol/l bilirubin (fig. 1). The cusum-test showed no significant deviation from linearity. The coefficient of variation was r = 0.9999.

Precision

The determination of the within-run precision was performed in three concentration ranges with low, medium and high bilirubin values, resulting in coefficients of variation between 0.37 – 0.76% (tab. 2). The between-run precision determined in four control sera over a period of 15 days resulted in coefficients of variation (CV %) between 0.51 – 1.76% (tab. 3).

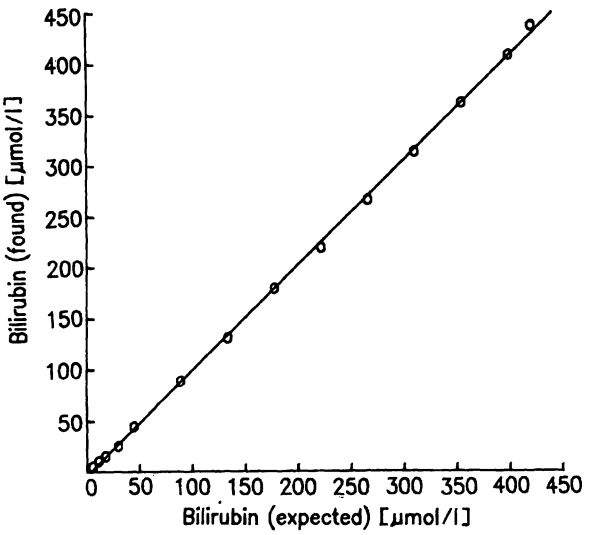

Fig. 1. Linearity of the bilirubin determination in plasma up to 437 µmol/l bilirubin.
x-axis: expected value,
y-axis: measured value,
equation of the line:
$y = -2.4 + 1.026x$,
correlation coefficient $r = 0.9999$.

Tab. 2. Within-run precision. Human pooled plasma at three different bilirubin concentrations

Material	n	$\bar{x}$ [µmol/l]	s [µmol/l]	CV [%]
Plasma 1	16	17.04	0.13	0.76
Plasma 2	16	133.21	0.59	0.44
Plasma 3	16	249.30	0.93	0.37

Tab. 3. Between-run precision. Control materials at five different bilirubin concentrations

Material	n	$\bar{x}$ [µmol/l]	s [µmol/l]	CV [%]
Ultimate D,				
Level 1	15	22.88	0.404	1.76
Level 2	15	89.86	0.476	0.53
Level 3	15	141.8	0.730	0.51
Level 4	15	289.6	2.156	0.72
Decision 1	15	18.37	0.228	1.24

Accuracy

The accuracy of the method was checked by means of assaying control sera, and by recovery of spiked plasma samples as well as of 6 bilirubin standard solutions (see 'reagents'), and by method comparisons. After a period of 15 days we obtained very good coefficients of variation between 0.14 and 4.27% (tab. 4).

Tab. 4. Accuracy of the Dri-STAT® method, given as the average relative deviation from the assigned value

Material	n	Assigned mean ($\bar{x}$) [µmol/l]	Found mean ($\bar{x}$) [µmol/l]	Average deviation [%]
Ultimate D				
Level 1	15	23.9	22.88	4.27
Level 2	15	90.7	89.86	0.93
Level 3	15	142.0	141.8	0.14
Level 4	15	295.6	298.6	1.01
Decision 1	15	19.0	18.37	3.31

The recovery rates of plasma samples one and two were 97 and 99%, and of the 6 bilirubin standard solutions between 92 and 99%. The scatter diagram of the bilirubin determinations (n = 88) with the oxidase method in comparison with the *Jendrassik & Grof* method as the reference is given in figure 2. The equation of the line was $y = -1.859 + 1.011x$, the correlation coefficient $r = 0.998$. In the additional comparison of the Dri-STAT® and the 2.5-dichlorophenyl diazonium salt method 140 patients' samples, also representing normal and abnormal test results were split and bilirubin was determined. A good correlation ($r = 0.997$) was obtained (fig. 3) and the equation of the regression line was $y = -0.920 + 0.995x$. For the two method comparisons no significant differences from $b = 1$ were calculated, however, significant differences from $a = 0$ do exist for both the increments of a. The cusum-tests showed no significant deviation from linearity. The bilirubin oxidase method resulted in values about 1 µmol/l and 2.5 µmol/l lower than those measured with the *Jendrassik & Grof* and the 2.5-dichlorophenyl diazonium salt method, respectively.

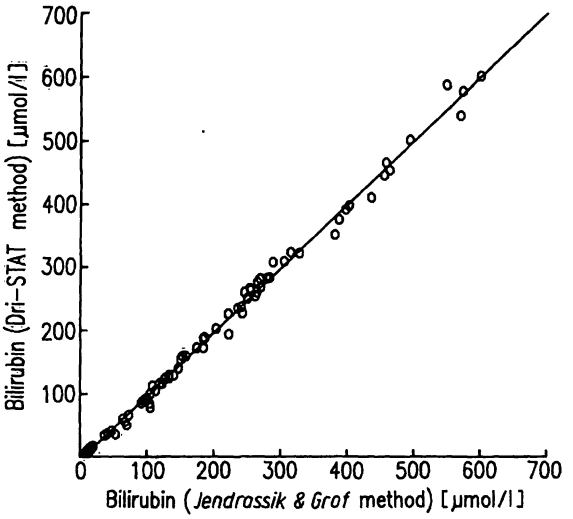

Fig. 2. Comparison of methods: *Jendrassik & Grof* (x) and the Dri-STAT® method (y) N = 88;
equation of the line $y = -1.859 + 1.011x$
correlation coefficient $r = 0.998$.

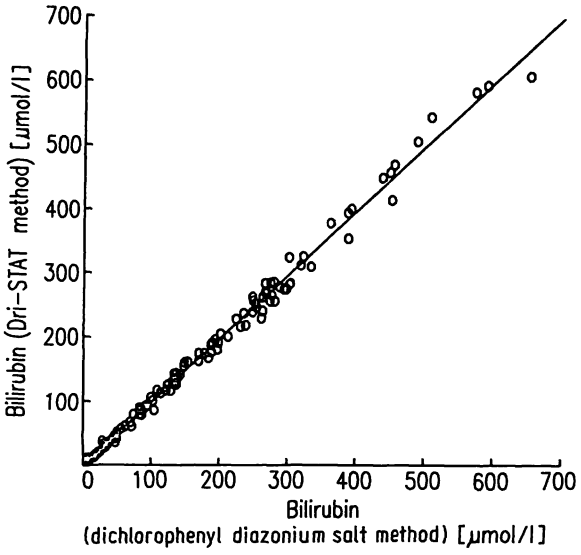

Fig. 3. Comparison of methods: 2.5-dichlorophenyl diazonium salt (x) and the Dri-STAT® method (y) N = 140; equation of the line y = −0.920 + 0.995x, correlation coefficient r = 0.997.

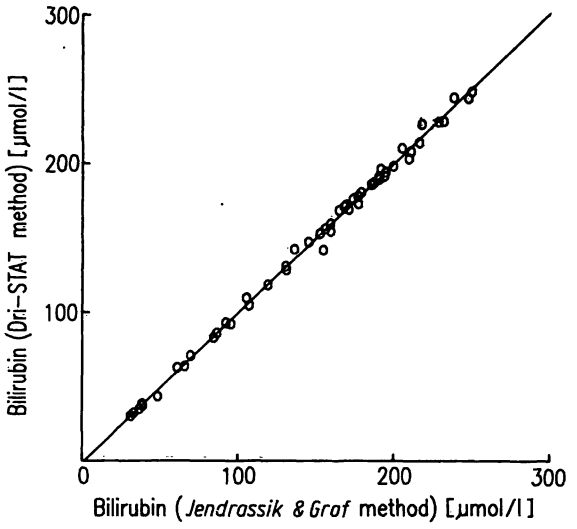

Fig. 4. Bilirubin determined in newborn sera with the *Jendrassik & Grof* (x) and the Dri-STAT® method (y) N = 53; equation of the line: y = −1.043 + 1.005x, correlation coefficient r = 0.998.

Detection limit

In order to ascertain the detection limit a solution of spectroscopically bilirubin-free bovine serum albumin (50 g/l) was determined (n = 16). The detection limit was calculated according to *Kaiser* (16) and lay at 0.3 µmol/l bilirubin.

Carryover

To assess carryover three successive aliquots of a plasma sample with high bilirubin concentration (240 µmol/l) were followed by three aliquots of a plasma with low bilirubin (7.3 µmol/l). This procedure was repeated ten times. A relative percentage carryover (carryover coefficient) of 0.042% was calculated according to *Haeckel* (17), and an overall maximal carryover of 0.2 µmol/l bilirubin into the sample with low bilirubin was calculated by the method of *Passey* and coworkers (18).

Bilirubin determination in sera from newborns and patients with chronic renal failure

We determined bilirubin in sera from 53 newborns with the Dri-STAT® method in comparison with the *Jendrassik & Grof* method. Corresponding results were obtained over a wide range (fig. 4). The equation of the straight line was y = −1.043 + 1.005x, the correlation coefficient r = 0.998. Significant differences from b = 1 and a = 0 could not be detected. The cusum-test showed no significant deviation from linearity.

Bilirubin was determined in 37 sera from uraemic patients with chronic renal failure (serum creatinine in the range of 681 − 1432 µmol/l) with the Dri-STAT® method in comparison with the *Jendrassik & Grof* reference procedure. Additionally the 2.5-dichlorophenyl diazonium salt method was used. The calculated means of the bilirubin determinations obtained by the Dri-STAT® method, the *Jendrassik & Grof*, and the 2.5-dichlorophenyl diazonium salt method were $\bar{x}$ = 11.0 µmol/l, $\bar{x}$ = 13.4 µmol/l and $\bar{x}$ = 27.2 µmol/l, respectively.

Interferences

We investigated the influences of some metabolites possibly responsible for the positive bias of the results from the 2.5-dichlorophenyl diazonium salt method in uraemic patients, anticoagulants, free haemoglobin, hypertriglyceridaemia, drugs and diagnostic substances.

The addition of the 13 metabolic substances aforementioned in 'materials' did not cause any bias of the bilirubin values obtained with the Dri-STAT® method. However, the metabolite 3-indoxylsulphate (indican) markedly increased the bilirubin values of the 2.5-dichlorophenyl diazonium salt method, resulting in an apparently total bilirubin of about 98 µmol/l for each 1 mmol/l of added indican.

The influence of in-vitro anticoagulants and a glycolysis inhibitor at concentrations normally found in plasma, was studied using aliquots of a plasma sample

containing 12.2 µmol/l bilirubin. No obvious deviation from this value was caused by EDTA, NH_4-heparinate, sodium citrate (1 part sodium citrate solution + 9 parts plasma sample as well as 1 part sodium citrate solution + 4 parts plasma sample) or sodium fluoride.

The addition of haemolysate to two plasma samples with different bilirubin concentrations caused decreasing bilirubin values dependent on increasing haemoglobin concentrations (tab. 5). The apparent decrease of the bilirubin concentrations is less in the sample with the higher bilirubin value.

Investigation of the influence of hypertriglyceridaemia showed only slight differences of the results obtained before and after delipidation with Frigen® in the range up to 12.3 mmol/l triacylglycerols (tab. 6). The average difference after Frigen® treatment was 0.15 mmol/l (= 4.1%) in the samples with low bilirubin and 1.5 mmol/l (= 4.6%) with elevated bilirubin.

Tab. 5. Influence of free haemoglobin on the Dri-STAT® method; c_H = apparent bilirubin concentration caused by haemoglobin interference, c_0 = bilirubin concentration without haemoglobin interference

Haemoglobin added [g/l]	Sample 1 Bilirubin		Sample 2 Bilirubin	
	measured [µ/l]	$\frac{c_H}{c_0}$	measured [µmol/l]	$\frac{c_H}{c_0}$
0	23	1.0	328	1.0
0.16	22.2	0.964	325.3	0.991
0.20	22.0	0.955	325.1	0.991
0.33	21.8	0.945	323.9	0.987
0.41	21.5	0.931	321.3	0.979
0.90	21.0	0.905	317.1	0.966
1.13	20.4	0.873	315.2	0.951
1.80	19.9	0.845	306.1	0.928
2.25	19.7	0.833	304.5	0.923
3.60	19.1	0.796	290.4	0.871
4.50	18.6	0.764	281.1	0.834

Tab. 6. Influence of hypertriglyceridaemia on determination of low and elevated bilirubin concentrations in serum before (a) and after (b) Frigen®-treatment

Sample	Triacylglycerols [mmol/l]	Low bilirubin [µ/l]		Elevated bilirubin [µmol/l]	
		a	b	a	b
1	2.85	3.2	3.3	32.6	34.4
2	3.75	3.4	3.0	31.1	33.0
3	4.95	3.5	3.6	31.3	31.0
4	5.20	3.8	4.2	33.7	34.4
5	5.35	2.9	3.2	31.3	30.8
6	7.15	4.5	4.5	35.2	36.2
7	8.05	3.8	3.6	32.9	35.4
8	9.05	3.3	3.7	29.9	32.5
9	10.05	5.7	5.9	33.5	35.4
10	12.3	2.7	3.3	29.5	33.3

The influences of drugs and diagnostics were investigated using aliquots of a plasma sample with 38 µmol/l bilirubin. The following pharmaceuticals at the concentrations shown in parentheses (g/l) were investigated:

Ampicillin (2.0),
Flucloxacillin (5.0),
Vancomycin (0.5),
Doxycyclin (0.1),
Piperacillin (10.0),
Cefuroxim 750 (0.75),
Gentamicin (0.2),
Imipenem (0.5),
Amikacin (1.0),
Tobramicin (0.2),
Dextran 40 10% (50.0),
Iopamidol (41.0),
iotalaminic acid 70 (14.0),
ioxaglinic acid (12.0),
sodium iopodate (3.0) and
acetylosalicylic acid (0.5).

No obvious influence on the bilirubin oxidase method could be detected.

Discussion

In clinical-chemical laboratories, enzymatic methods for substrate determination are often used, because they are specific and, in most cases, easy to handle, e. g. the determinations of glucose, ammonia, urea and creatinine.

Recently a new enzymatic method for the determination of bilirubin in serum or plasma has been described based on the oxidation of bilirubin by bilirubin oxidase (EC 1.3.3.5) (6, 7).

The results presented in our study confirm the previously published findings of the above mentioned group (9) and demonstrate an even better analytical accuracy.

The superior precision in different ranges and the lack of carryover can certainly also be ascribed to the pipetting system and the washing and drying procedure of the Hitachi 704. The results of the within-run as well as the between-run imprecision are very satisfactory. With respect to accuracy, assessed by measuring control sera, and the recovery of spiked plasma samples as well as of bilirubin standard solutions, and by method comparisons, the bilirubin oxidase method fulfils the usual analytical and clinical requirements very adequately.

As the comparison with the *Jendrassik & Grof* method points out, a good correlation (r = 0.998) exists, and the mean of concentrations measured enzymatically is only about 1 µmol/l lower. Hence we can confirm the correlation results of *Ladouceur* et al. (19, 20) who calculated a slope of b = 0.988, an intercept of a = −0.150 and a correlation coefficient of r = 0.991.

With view to the accuracy of the bilirubin oxidase method, the question arises as to whether the four fractions (α−δ) of total bilirubin, especially the so-called δ-fraction, are completely determined in the course of various hepatobiliary diseases.

To begin with, the good accuracy of the *Jendrassik & Grof* method, which we used as the reference, has been proved several times. In an extended study the serum bilirubin of adult patients with hyperbilirubinaemia was investigated by *Weiss* et al. (21), using HPLC for the four separate fractions, and the *Jendrassik & Grof* method for total bilirubin. In a similar study using the same two methods, total bilirubin and its fractions were determined in sera from paediatric patients (22). *Lauff* et al. (23) ascertained a good correlation of total bilirubin values by the *Jendrassik & Grof* procedure with those of a HPLC method. The latter authors found that the δ-fraction can account for up to 0.92 of the total bilirubin in pathological sera, so erroneous results may be obtained in the spectrophotometric assay for direct but not total bilirubin. In this context it must be mentioned that similar bilirubin values can be obtained by HPLC and the *Jendrassik & Grof* method, only if the optimal reaction conditions for the *Jendrassik & Grof* procedure are used, as pointed out recently (12, 24). From all these findings the conclusion can be drawn that the *Jendrassik & Grof* method, as proposed by *Doumas* et al. (12), is well suited for measuring total bilirubin accurately, regardless of subcomponent composition. Regarding the excellent correlation of the bilirubin oxidase method with the *Jendrassik & Grof* method, we believe the new enzymatic method to be a very reliable procedure.

Furthermore, the method is suitable for measuring bilirubin in newborn sera over a wide range, and it performs as well as the *Jendrassik & Grof* method (r = 0.998, b = 1.005, a = −1.043). This circumstance might be of great interest for paediatric hospitals, because the method needs a sample volume of only 15 µl, and the decision range for application of phototherapy or exchange transfusion is clearly included by the linearity range, which extends to 437 µmol/l bilirubin.

Depending on the analytical method, haemolysis interferes to a greater or lesser extent with the spectrophotometric determination of bilirubin. Hitherto, the procedure least subject to interference by haemolysis has been the *Jendrassik & Grof* procedure, published as the reference method by *Doumas* et al. (12); addition of 2.5 g/l haemoglobin to a serum sample containing 171 µmol/l bilirubin caused an apparent decrease in bilirubin of less than 2%; when ascorbic acid was present in the reaction mixture no decrease was observed. Other commercially available modifications of the *Jendrassik & Grof* method as well as the various dichlorophenyl diazonium salt procedures are influenced by free haemoglobin. After the addition of 2.5 g/l free haemoglobin to a serum sample containing 71 µmol/l bilirubin, *van der Woerd* et al. (25) found an apparent decrease in the bilirubin concentration of 35% by the 2.5-dichlorophenyl diazonium salt method, and about 12% by the *Jendrassik & Grof* method. From the results in table 5, a serum bilirubin value of 71 µmol/l determined with the Dri-STAT® method will be decreased by about 15%, if 2.5 g/l free haemoglobin are added. The apparent decrease of bilirubin is directly related to the amount of free haemoglobin and dependent on the bilirubin concentration (9), as confirmed in the present study (tab. 5), i. e. low bilirubin values are more influenced than high values.

These findings have a bearing on the determination of bilirubin in newborn sera by means of the Dri-STAT® method. *Meites & Lin* (26) measured haemolysis in samples from 417 children, of whom 176 were less than 14 days old. They found a mean value of 0.4 g/l haemoglobin for the newborns (0−13 days); 73% of these values were below 0.5 g/l, whereas less than 5% were above 1 g/l. The highest value seen was 1.47 g/l. Taking these findings into consideration together with our results in table 5, one can expect an apparent bilirubin decrease of 2−6% in the range of 250−330 µmol/l serum bilirubin, if the serum also contains 0.4−1 g/l haemoglobin.

Falsely positive bilirubin values determined with the 2.5-dichlorophenyl diazonium salt method in sera from patients with chronic renal failure are well known and have also been described for the 2.4-dichlorophenyl diazonium salt method (27, 28). The only metabolite found until now in uraemic sera, interfering with the dichlorophenyl diazonium salt methods, is indican. In contrast, the accuracy of the bilirubin oxidase method remained unaffected, as confirmed by our results with the *Jendrassik & Grof* method, which has previously been shown to be free

from interference by indican (27). Other possible sources of interference had no effect on the bilirubin oxidase method.

The influence of hypertriglyceridaemia (up to 12 mmol/l) on the enzymatic bilirubin determination is very small, so that from a practical point of view a clearing treatment is unnecessary, although the difference between the matched pairs with elevated bilirubin was statistically significant. The high dilution of the small volume in the Hitachi 704 might be the reason for the slight interference.

In conclusion, the bilirubin oxidase method can be recommended for application in clinical-chemical laboratories.

Acknowledgement

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